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SHORT COMMUNICATION

Development of novel microsatellite markers for the spider genus *Loxosceles* (Sicariidae) using next-generation sequencing

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Abstract. We report the step-by-step process of developing *de novo* microsatellite (SSR) loci in two *Loxosceles* spider species. We used reads obtained with next-generation sequencing (Roche 454) to select hundreds of potentially-amplifiable SSRs. After testing amplification and cross-amplification, we characterized 18 SSRs, 11 of which were polymorphic in *Loxosceles rufescens* (Dufour 1820) and seven of which were polymorphic in *L. sp.* Fuerteventura - Lanzarote. This method is a relatively fast and economic procedure for the development of fast-evolving nuclear markers in spiders.

Keywords: 454, nuclear markers, cross-amplification, Mediterranean, Canary Islands

Microsatellites (SSRs: simple sequence repeats) are popular codominant genetic markers used in many areas of research, including molecular ecology and population genetics. They consist of tandem repeats of very short nucleotide motifs (1–6 bases long). One property that makes SSRs attractive for evolutionary studies is their high mutation rate (Guichoux et al. 2011). However, the technical and economic effort required for developing *de novo* SSRs in organisms for which no or few genomic resources are available (the so-called non-model organisms) has, until recently, prohibited wide implementation. The recent emergence of next-generation sequencing technologies has reduced the economic and technical difficulties associated with developing SSRs (Santana et al. 2009), and has boosted their usage in a wide range of organisms, including spiders (Esquivel-Bobadilla et al. 2013; Parmakelis et al. 2013), a group in which the development and application of microsatellites has been limited (Brewer et al. 2014).

In this study, we focused on spiders of the genus *Loxosceles* Heineken & Lowe 1832 (Araneae: Sicariidae) from the Mediterranean Basin and the Canary Islands. *Loxosceles rufescens* (Dufour 1820) is considered cosmopolitan (World Spider Catalog 2014), but is native to the Mediterranean (Gertsch 1967; Duncan et al. 2010; Planas et al. 2014). In this region, several deep mitochondrial lineages have been detected (Duncan et al. 2010; Planas et al. 2014), some of which lack geographic structure as a consequence of the confounding effects of human-mediated transportation. Recently, Planas and Ribera (2014) discovered an endemic group of seven lineages of *Loxosceles* spiders in the Canary Islands. The two easternmost islands in this archipelago, Fuerteventura and Lanzarote, harbor one of these identified lineages. Despite the relatively impoverished fauna of Fuerteventura and Lanzarote, these two islands, together with the surrounding islets, have been shown to be ideal systems to study phylogeographical processes (i.e., Bidegaray-Batista et al. 2007; Macías-Hernández et al. 2013). Here, we acquired fast-evolving nuclear loci for the study of fine-scale evolutionary processes in the *Loxosceles* species endemic to Fuerteventura - Lanzarote (hereinafter *Loxosceles* sp. FV-LZ), and for contrasting the mitochondrial patterns observed within *L. rufescens* across the Mediterranean Basin (Planas et al. 2014).

We used next-generation sequencing to obtain SSRs and describe the step-by-step process from DNA extraction to characterization of selected markers. Genomic DNA was extracted from the legs of three specimens of *Loxosceles,* two of which belong to two different evolutionary lineages (A6 and B3; Planas et al. 2014) within *L. rufescens,* and a third belonging to *Loxosceles* sp. FV-LZ, using the SpeedTools Tissue DNA Extraction Kit (Biotools) following manufacturer's protocols. We conducted pyrosequencing on a Roche Life Science 454 GS-FLX System at the University of Barcelona's Scientific-Technical Services. Roche 454 is a next-generation sequencing technology that obtains larger average fragment sizes, thus increasing the probability that the fragments containing SSRs have flanking regions to enable primer design. We pooled samples using individual multiplex identifiers (MIDs), together with an *Echinaster sepositus* sample (Garcia-Cisneros et al. 2013), within half a plate because physical separation decreases the overall number of sequences obtained. We acquired a total of 143,708 reads with a mean length of 341.86 bp for *Loxosceles* sp. FV-LZ, 45,377 (mean length 313.91 bp) for *L. rufescens* A6, and 195,081 (mean length 346.24 bp) for *L. rufescens* B3.

Raw data were processed with the Roche's 454 pipeline using default settings for quality control and with seqclean (https://sourceforge.net/projects/seqclean/) to remove low quality sequences and contaminants. Sequence reads from duplicated loci and mobile elements were identified in iQDD (Meglécz et al. 2010) using default parameters and were excluded from further analyses. We searched for reads with SSRs using iQDD, and retained those meeting a series of requirements suggested by Guichoux et al. (2011). Specifically, we looked exclusively for SSRs with perfect motif repetition, improving the probability that the SSRs follow a stepwise mutation model. We searched for SSRs with a minimum of 11 repeats in dinucleotides and eight repeats in tri-, tetra-, penta- and hexanucleotides, but no more than 16 repeats in both cases. Primers for selected SSRs were designed with the software PRIMER 3 (Rozen & Skaletsky 2000) included in iQDD. We avoided designing primers in flanking regions containing short repeats (e.g., nanosatellites), and we selected putative PCR products between 90 and 500 bp in length. Among all possible primer combinations for each SSR, we kept those with better evaluation based on the penalty score of the primer pairs after applying stringent parameters to ensure amplification (i.e., no primer-dimer interaction, similar annealing temperature, GC primer end content, and primer end stability). The number of reads containing SSR and the number of those with suitable flanking PCR-primer sites are shown in Table 1. Dinucleotides were the most frequent SSR, followed by tri-, tetra-, penta- and hexanucleotides (Table 1). Even after applying stringent parameters for SSR selection, we obtained over 800 SSRs that met the requirements specified above. We should note that relaxed selection criteria rigor (e.g., allowing for a minimum number

Table 1.—Number of reads containing SSRs and number of potentially amplifiable SSRs. Individual cells in the table record the number of reads obtained from each individual (*Loxosceles* sp. FV-LZ / *L. rufescens* A6 / *L. rufescens* B3).

	Dinucleotides	Trinucleotides	Tetranucleotides	Pentanucleotides	Hexanucleotides
Reads containing SSRs	3525/961/4261	334/141/673	38/34/181	3/0/6	0/0/1
Reads with potentially amplifiable SSRs	327/107/206	37/21/87	1/1/7	1/0/5	0/0/0

of eight tandem repeats) would have increased substantially the number of yielded SSRs. We selected 58 among the hundreds of candidate SSRs, considering the length of the expected PCR product. We then tested their amplification and cross-amplification success in eight individuals, four from *L. rufescens* and four from *L.* sp. FV-LZ. That is, we tested SSRs obtained from reads of *L. rufescens* for amplification in *L.* sp. FV-LZ individuals and *vice versa*. Of the 58 SSRs tested, 40 were rejected because PCR amplification was unsuccessful.

We retained the 18 SSR loci with higher amplification success and labeled the forward primers with fluorescent dye. We tested for polymorphism using 38 *L. rufescens* individuals from four different localities, and 16 *Loxosceles* sp. FV-LZ individuals from four different localities. We conducted PCR reactions in a final volume of 10 μL using Biotools *Pfu* DNA Polymerase (Biotools). Annealing temperatures ranged between 42° and 58° C for all primer pairs. We pooled PCR products according to dye type and expected allele size ranges, and genotyped them in an ABI 3730XLs automated sequeneer at Macrogen (Seoul) with the internal size standard 500 LIZ. We used the Microsatellite Plugin 1.3 in Geneious 6.1.6 (Biomatters) for allele calling. For each locus, the primer sequences, number of alleles (N_a), and observed (H_O) and expected (H_E) heterozygosity are listed in Table 2.

All but one SSR were polymorphic for at least one of the two species analyzed. One SSR (ME083) obtained from *L. rufescens* reads

Table 2.—Characteristics of 18 microsatellite loci, tested with 38 samples of *Loxosceles rufescens* from four different localities, and 16 samples of *Loxosceles* sp. FV-LZ from four different localities. Locus name, accession number, repeat motif and primer sequences (F: forward, R: reverse) are listed for each locus. In the last four columns of the table, *L. rufescens* data are presented in the first row for total number of alleles, allele size (bp), expected heterozygosity (He) and observed heterozygosity (Ho), and *Loxosceles* sp. FV-LZ in the second row.

Locus	Accession number	Repeat motif	Primer sequence (5′–3′)	Total number of alleles	Allele size (bp)	Ho	He
ME012	KM879453	(AGAT)	F: GTGGGTGGTCCATTGATAGG	8	137–165	0.57	0.77
			R: TTTAACAAGACGCAGCGAAA	-	-	-	-
ME031	KM879448	(AAAT)	F: AAACTTCGATTTATTTTGTTTCTTG	4	89–109	0.19	0.66
			R: AAATGTCTGGCGGATCAGAA	-	-	-	-
ME034	KM879450	(AAAT)	F: CGTCTGCAGTGTGAACGG	6	93–149	0.47	0.71
			R: ATATGTGCTTTTGCGCCTGT	-	-	-	-
ME064	KM879451	(AAAT)	F: TCTGTAAATGGATTCTCATCTGTTG	2	151–155	0.13	0.12
			R: TCGTCCAACCATCCTCTTTC	-	-	-	-
ME067	KM879446	(AGAT)	F: TGTGATGTACCTGCGTTCGT	4	142–160	0.11	0.10
			R: GCAAGATCAACCCACAACCT	-	-	-	-
ME077	KM879454	(AAACT)	F: TATGTAATCACCGGGGTTGG	3	152–177	0.21	0.55
			R: CGTGCAATCTGGTTAACTTCG	-	-	-	-
ME083	KM879445	(ACACT)	F: TAGGGAATGGAATGGCAGAC	1	160–160	0	0
			R: TTTGCAGATTTGATCTGGGAC	1	163–163	0	0
ME088	KM879449	(AAAT)	F: AGCGTTGATACAGGTGGTCC	3	208–254	0.10	0.59
			R: TCACTGCACAGTGTAAAGCCA	-	-	-	-
ME103	KM879452	(AAT)	F: TTAGCGACCTTCCCTGTCAC	6	262–280	0.34	0.73
			R: TGGTAAACGGGAGGACTAGG	-	-	-	-
ME113	KM879447	(AAT)	F: AACCTGAAGGGCTGATGAAT	6	75–96	0.37	0.78
			R: CAGGAGCAGGATGCCATATT	-	-	-	-
CA001	KM879461	(AAT)	F: ATGTATCACGCGCCTTTTG	-	-	-	-
			R: GTTGTCTGGAGCAAACAGCA	5	75–93	0.60	0.72
CA003	KM879460	(AAT)	F: TGTACCAGGGGCTGGTCTAA	-	-	-	-
			R: CATACGTGGTGGCAGCATAC	5	66–92	0.28	0.73
CA027	KM879457	(AAGTG)	F: TACCACAAGGGGAGAATCCA	3	103–113	0.39	0.32
			R: AAGCCAGAGGTGCAATTGTT	10	132–182	0.40	0.79
CA030	KM879462	(AAT)	F: AGGTGTGGCACTACCGTTTT	-	-	-	-
			R: CAAATGAGCATTCAACCTCG	7	133–157	0.46	0.70
CA038	KM879458	(AAT)	F: ATGTTTGAGGGGTCTCGTTG	-	-	-	-
			R: ACATGATGCCCCACGATAAT	4	272–284	0.93	0.69
CA105	KM879455	(AC)	F: TAAATAACCTGATATCGGATCTATGAC	-	-	-	-
			R: AAAGTATATCGGACAAACATCCAACC	5	255–267	0.75	0.75
CA238	KM879459	(AG)	F: GGCACCCCAGACTAACAAGA	1	233–233	0.00	0.00
			R: ACCTCTGGCACGAATACACC	4	221–231	0.93	0.69
CA243	KM879456	(AT)	F: AATAACGGAGACCGTGCAAC	5	225–279	0.68	0.64
			R: CCTCCAGTATCCGAAGACGA	-	-	-	-

amplified successfully in *Loxosceles* sp. FV-LZ individuals, although it was monomorphic in both species. Three SSRs obtained from *L.* sp. FV-LZ reads amplified successfully in *L. rufescens*, and was monomorphic in one locus (CA238) and polymorphic in the other two loci (CA027 and CA243). In total, 11 polymorphic SSRs were developed for *L. rufescens* and seven for *L.* sp. FV-LZ.

Results from this study suggest that next-generation sequencing is an efficient and cost-effective procedure for the fast development of microsatellite loci in spiders. Despite the close phylogenetic relationship of the two species used in this study (Planas & Ribera 2014), the cross-amplification rate for the microsatellites was low. The few SSRs that cross-amplified successfully were found to be monomorphic or less polymorphic in the species from which they were not initially obtained (except for CA243). Thus, we advise developing specific microsatellites for each target species. We obtained thousands of reads by sequencing three *Loxosceles* specimens in half a Roche 454 plate, and we used a fast bioinformatic pipeline applying stringent selection criteria to identify hundreds of potentially amplifiable SSRs. Although 454 sequencing was preferred for the longer read lengths obtained which facilitates the design of PCR primers, a similar approach for SSR development has been successfully implemented using alternative, more cost-effective sequencing technologies (i.e., Illumina) (Castoe et al. 2012, but see Drechsler 2013).

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